

Monitoring of mite *Dermatophagoides farinae* allergen-specific IgG and IgG subclass distribution in patients on immunotherapy

Einarsson R, Dreborg S, Hammarström L, Löfkvist T, Smith CIE, Svensson G. Monitoring of mite *Dermatophagoides farinae* allergen-specific IgG and IgG subclass distribution in patients on immunotherapy. *Allergy* 1992; 47: 76–82.

House dust mite *D. farinae* and *Der f II*-specific IgG and IgG subclass responses were evaluated in 32 adults with perennial rhinitis undergoing immunotherapy for 1 year by means of IgG-RAST and ELISA. The ELISA method, which is based on subclass-specific monoclonal antibodies, could detect 0.5–1.5 ng/ml of specific antibodies. *D. farinae* and *Der f II*-specific IgG₄ antibodies increased continuously as immunotherapy proceeded, while total IgG and IgG₁ antibodies reached a plateau value 6 months after the start of immunotherapy, followed by a slow decrease during maintenance therapy. During the early phase of treatment the concentration of IgG₁ and IgG₄ antibodies rose, even though the increase of IgG₄ antibodies dominated. The positive clinical outcome as measured by the decrease in conjunctival sensitivity was associated with an increased ratio of specific IgG₄/IgG₁ antibodies as well as the magnitude of the IgG₄ subclass response. Quantitation of IgG subclass antibodies in patients undergoing immunotherapy may be of some clinical value, but the clinical usefulness needs to be demonstrated for each type of allergen and possibly also for each assay system.

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Key words: *Der f II*; IgG subclasses; immunotherapy; mite allergy.

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Accepted for publication 4 April 1991

House dust mite allergy is generally considered to be an important cause of allergic rhinitis in north European patients with perennial allergy (14, 20). The treatment of dust mite-associated chronic rhinitis has been based on pharmacological agents, avoidance of mites, or immunotherapy (IT).

Immunotherapy has been widely used in the treatment of different allergic disorders, particularly pollinosis and rhinitis (25, 34). On the other hand, relatively few controlled IT studies with documented clinical efficacy have been reported using house dust mites (5, 30).

During IT with potent allergens common immunological changes have been noted, such as initial rise in IgE, followed by gradual decrease after several years of active therapy, increase in the level of blocking allergen-specific IgG antibodies or decrease in basophil cell reactivity to the allergen (26). The amount of IgG antibodies formed during IT appears to be related to the amount of antigen administered, but only a minor fraction of the induced IgG antibodies may be of clinical significance. A marked increase in anti-*Der p I* IgG antibodies has been

reported during mite IT, with no change or only a slight reduction in the level of specific IgE antibodies (3, 4).

The IgG production at exposure to proteins is mainly of the subclass IgG₁, but to some extent also IgG₄ antibodies are produced. Specific IgG₄ antibody responses have been detected in patient sera where the offending allergens are food proteins, inhaled antigens or venoms (2, 23, 31). Many attempts have been made to identify the IgG subclass acting as possible blocking antibodies and the IgG₄ subclass has been proposed as the relevant one. However, some authors emphasize the importance of IgG₁ antibodies rather than those of IgG₄ (7, 9).

We studied the total IgG and IgG subclass response in sera from patients undergoing IT with a standardized allergen preparation of *D. farinae* using IgG RAST with all mite proteins on the solid phase and ELISA with all mite proteins on the well surface or purified Ag 19/20 (*Der f II*). Further, an attempt was made to correlate the IgG subclass response to the change in clinical sensitivity as measured by conjunctival sensitivity.

Material and methods

Patients

Thirty-two patients with documented allergic rhinitis to house dust mites were randomized with 21 patients (mean age 28 years) in a group for active treatment and 11 patients (mean age 24 years) in a control group (16). All patients had a clinical history of perennial mite rhino-conjunctivitis, positive skin prick test (SPT) with house dust mite *D. farinae* (100 000 biological units, BU/ml) and positive RAST (RAST class 3 or 4). The patients were given subcutaneous injections with purified house dust mite preparations. Doses were increased from 50 BU/ml to 100 000 BU/ml over 13 weeks and then given with the individual top dose at 6–8 weeks interval up to 1 year. The maintenance dose ranged from 20 000–100 000 BU/ml. There was a significant improvement in the group on *D. farinae* therapy regarding subjective evaluation ($p < 0.01$), skin prick test ($p < 0.0001$) and conjunctival provocation test ($p < 0.001$) (16). Serum was drawn initially and before each injection and was kept frozen until analysed. Blood samples from the control group were drawn initially and after 3 and 12 months. Conjunctival provocation test (CPT) was performed according to the method described by Möller et al. (21). The starting dose was 100 BU/ml, and increased with half log-steps up to 32 000 BU/ml (16). Serum from 2 patients in the treated study group were not analysed by *in vitro* assays due to insufficient amounts of serum available.

Allergen preparations

A purified, well-characterized, biologically standardized and freeze-dried preparation of mite *D. farinae* (Pharmalgen, Pharmacia Diagnostics AB, Uppsala, Sweden) was used as antigen in the *in vitro* studies (6). The same mite material (delivered in 4 concentrations 100 BU/ml to 100 000 BU/ml) was used for immunotherapy and *in vivo* diagnosis (SPT, CPT). One of the clinically most important *D. farinae* allergens, Ag 19/20, was purified from isolated and crushed *D. farinae* mites by a combination of gel filtration on Ultrogel AcA-54 and cation exchange chromatography on the Mono S HR 5/5 (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the procedure described by Smedstad et al. (19). According to the nomenclature recommended by WHO a purified *D. farinae* allergen previously termed Ag 19/20 is *Der f II* (18, 29). *Der f II* was only used in ELISA assays.

Specific IgG by RAST

Total specific IgG antibodies against house dust mite allergens (*D. farinae*) were measured with Phadebas IgG RAST (Pharmacia Diagnostics AB). The results were expressed as percentage of the fraction activity bound (% T). All tests were performed in duplicate with patient sera diluted 1/200.

ELISA for quantification of IgG subclasses

IgG subclasses were quantitated by an ELISA method essentially as described previously (28). Monoclonal antibodies against IgG₁, IgG₂, IgG₃ and IgG₄ were purchased from Unipath, Bedford, England (anti-IgG₁ (JL 512); anti-IgG₂ (GOM 1); anti-IgG₃ (ZG 4); anti-IgG₄ (GB7B and RJ4)). Purified whole mite extract or an isolated *D. farinae* allergen *Der f II* (5 µg/ml) in 0.1 mol/l PBS, pH 7.2, was incubated in each well of a microtiter plate (Dynatech Laboratories) overnight at 37°C. The mite antigen coated plates were washed 4 times and the patients' sera diluted 1:100 in PBS-buffer. After incubation overnight followed by several washings, monoclonal mouse anti-human IgG subclass antibodies were added. The plates were again washed and incubated for 4 h with alkaline phosphatase-conjugated rabbit anti-mouse IgG diluted 1:1000. After incubation overnight in room temperature, followed by several washings a substrate (p-nitrophenyl phosphate) was added and read at 405 nm at 10 min intervals. The absorbance values for specific antibodies could be compared with the figures obtained for fixed amounts of myeloma proteins (assayed in parallel) of the different subclasses giving a semi-quantification of the allergen-specific IgG subclass antibody content. The assay is dependent on the proven specificity of anti-IgG subclass reagents used.

Statistical analysis

The variability in RAST IgG and subclass ELISA was studied by estimating the mean and 95% confidence limits or 2 SEM. For evaluation of changes in specific antibodies within and between groups the Student's t-test was used. Correlation analysis between serological parameters was done by calculating Pearson or Spearman correlation coefficients.

Results

Total mite-specific IgG

RAST IgG was used to measure the content of allergen-specific IgG antibodies against house dust

mite antigens in serum samples collected at different time periods. Fig. 1 demonstrates the change in specific IgG during the course of *D. farinae* immunotherapy for the patients in the active group and in the control group versus time of treatment. The levels of specific IgG varied for the individual patients, but no significant difference was observed between the study groups at the start of IT. Patients treated with *D. farinae* allergen preparation showed a distinct increase in the level of mite-specific IgG. The highest antibody level was reached after 4 months of IT (Fig. 1) and thereafter there was a slow decrease of mite-specific IgG antibodies. After 12 months of treatment the total mite-specific IgG antibody level was still high and significantly higher than before treatment ($p < 0.05$). The patients in the control group showed a constant and low level of specific IgG antibodies during the treatment period. After 12 months of IT the treated group showed about 40% higher value of mite specific IgG antibodies than the control group ($p < 0.05$).

Spec. IgG

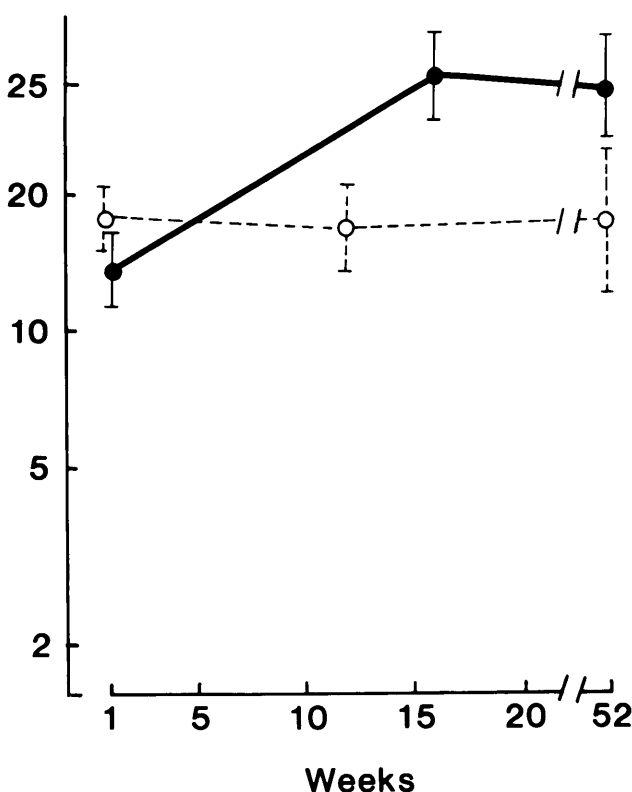


Fig. 1. House dust mite *D. farinae*-specific IgG measured with IgG-RAST and expressed as percent of activity bound for the patients on active treatment and the control versus time of treatment. Solid line: active group; broken line: control group.

IgG subclasses

The effect of immunotherapy was also monitored by an enzyme-linked immunosorbent assay measuring IgG subclass response towards a *D. farinae* mite allergen mixture and a major mite allergen *Der f II*.

For screening purposes we started by analysing all four IgG subclasses with respect to mite-specific IgG subclass response. In the first series of experiments purified whole *D. farinae* allergen mixture was used as antigen and adsorbed to the microtiter wells. The subclass response towards the *D. farinae* allergen mixture and *Der f II* was restricted to IgG₁ and IgG₄. We were unable to detect any change in IgG₂ or IgG₃ anti-*D. farinae* or anti-*Der f II* activity in either the treated patients or in the control group (results not shown).

Fig. 2 shows the geometric mean $\pm$ 2 SEM of the mite-specific IgG₁ and IgG₄ antibodies given as absorbance values at 405 nm. The IgG₄ specific antibodies increased rapidly in the treated study group and after 12 months the geometric mean was approximately 6-fold higher compared with the mite-specific IgG₄ level in the control group. The specific IgG₁ antibodies also showed a definite increase versus time of treatment, but this effect was not as pronounced as with IgG₄. Mite-specific IgG₁ antibodies peaked after 6 months and then decreased slightly. However, the active group showed about 2-fold higher IgG₁ antibody content compared with the control group after 1 year of treatment. All patients in the treated group and in the control had low and similar levels of mite-specific IgG₁ and IgG₄ antibodies from natural exposure prior to treatment.

Fig. 3 demonstrates the specific IgG₁ and IgG₄ antibody response to the clinically important *D. farinae* allergen *Der f II* in both study groups. There was no significant difference in the level of subclass antibody content between the groups for either IgG₁ or IgG₄ before the start of treatment. *Der f II*-specific IgG₁ showed a distinct increase (about 2.5-fold) after 6 months and then declined. After 12 months there was still a significant difference between the study groups compared with the pretreatment level. *Der f II*-specific IgG₄ antibodies increased approximately 4-fold during the first 6 months of treatment followed by a slight decrease up to 12 months. The IgG₁ antibody level increased significantly in the active group during the treatment period, and after 12 months the difference between the active and the control groups was about 5-fold. The IgG₁ and IgG₄ antibody levels in the control remained constant at the pretreatment level. We also tried to quantitate the amount of house dust mite-specific IgG₁ and IgG₄ subclasses in relation to the total amount of each subclass with the ELISA method. The content of IgG₁ and IgG₄ mite-specific subclasses in the

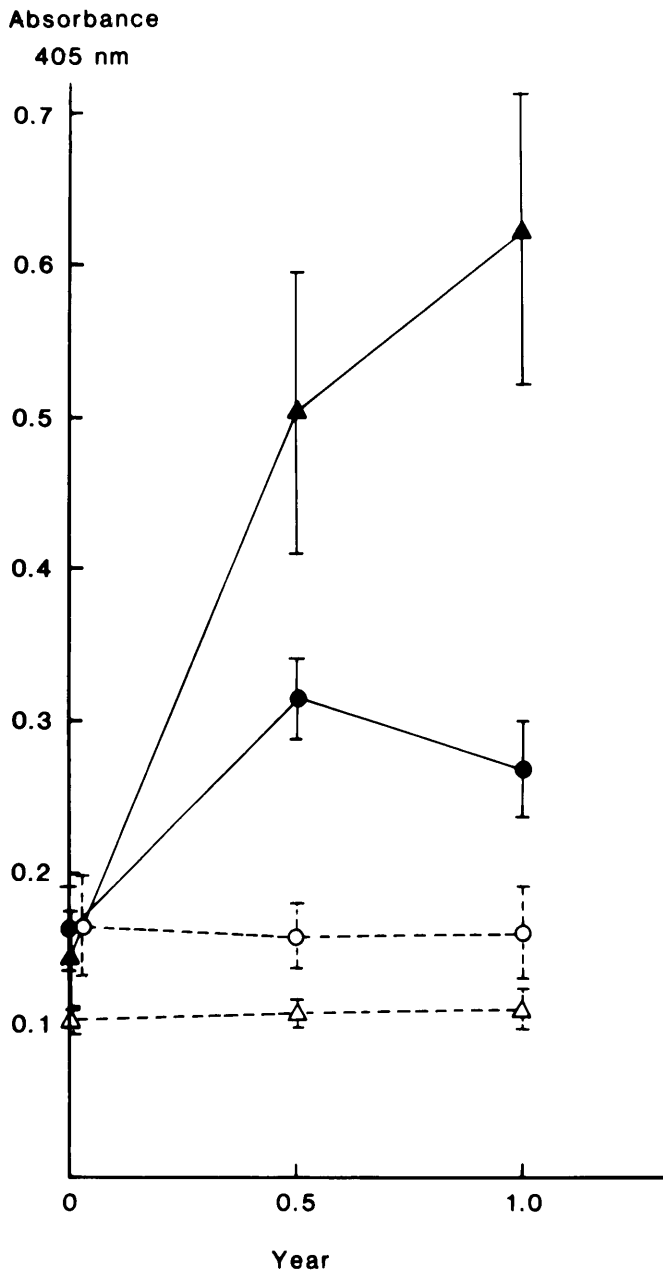


Fig. 2. Mean *D. farinae* allergen-specific IgG₁ and IgG₄ antibodies during immunotherapy versus time of treatment (●—●) IgG₁, active group; (▲—▲) IgG₄, active group; (○- - -○) IgG₁ control; (△- - -△) IgG₄ control.

treated patients was about 0.01% for IgG₁ and 0.5% for IgG₄ of the total subclass content. However, it must be borne in mind that the assay is at best semiquantitative.

Subclass ratios

The ratios between *Der f II*-specific IgG₄ and *Der f II*-specific IgG₁ as well as the ratio between mite *D. farinae* allergen-specific IgG₄ and IgG₁ are shown

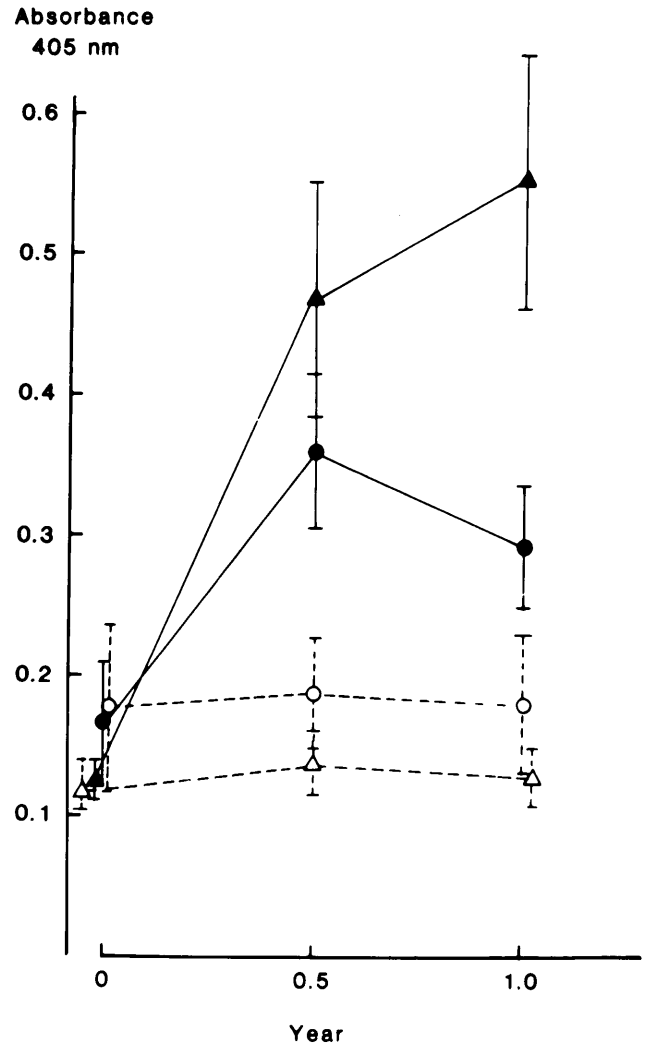


Fig. 3. Mean IgG₁ and IgG₄ antibody responses to a house dust mite allergen, *Der f II*, during immunotherapy versus time of treatment; (●—●) IgG₁ active group; (▲—▲) IgG₄ active group; (○- - -○) IgG₁ control; (△- - -△) IgG₄ control.

in Fig. 4. The calculated IgG subclass ratios for both *Der f II* and *D. farinae* allergen mixture increased in parallel during the treatment period for the treated patients, while the ratios for the untreated controls remained constant. The ratio IgG₄/IgG₁ with *D. farinae* allergen mixture as antigen showed a somewhat, but not significantly, higher value compared with the ratio IgG₄/IgG₁ for *Der f II* during the treatment period. Before treatment there was not a significant difference between the subclass ratios for the active group and the control. After 12 months of treatment a mean rise of 3.4-fold was observed in the ratio IgG₄/IgG₁ for the *D. farinae* allergen mixture and a rise of 2.6-fold for *Der f II* as antigen, respectively.

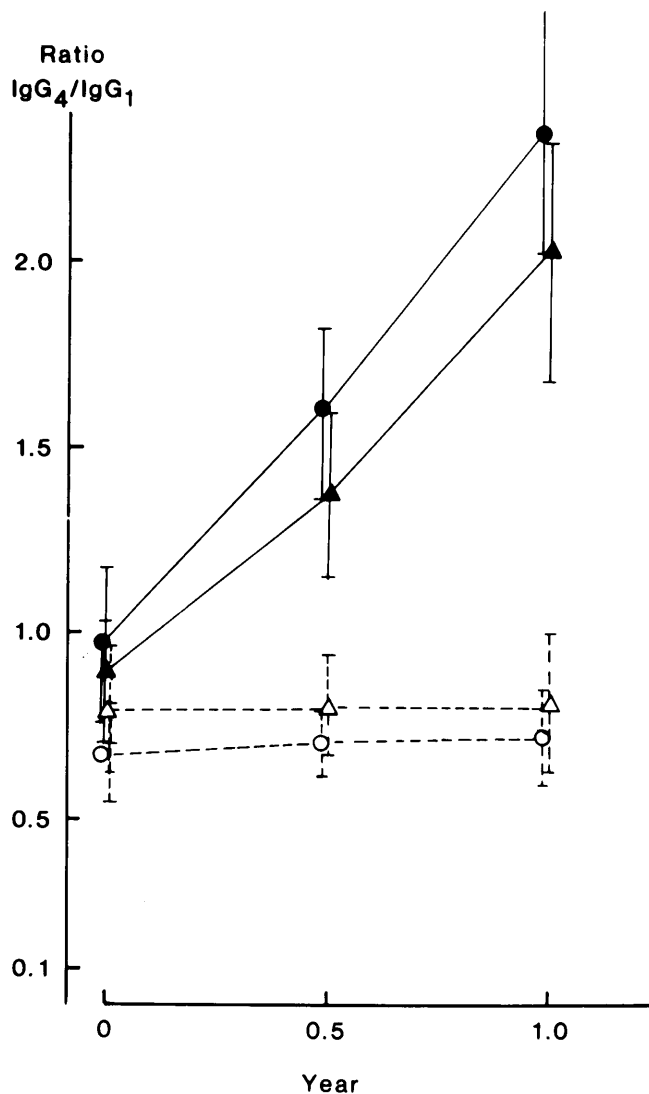


Fig. 4. Mean ratio IgG₄/IgG₁ to *D. farinae* allergens and *Der f II* during immunotherapy; (●—●) *D. farinae* active group; (▲—▲) *Der f II* active group; (○—○) *D. farinae* control; (△—△) *Der f II* control.

Subclass ratio versus clinical improvement

Fig. 5 demonstrates the changes in the ratio IgG₄/IgG₁ with *Der f II* as antigen in the ELISA assay, versus changes in the threshold conjunctival concentration for the individual patients on active treatment with *D. farinae* allergen preparation and control subjects. Most patients on immunotherapy showed a decreased conjunctival sensitivity and a higher antibody ratio IgG₄/IgG₁. Three patients on active treatment demonstrated an increased subclass ratio (IgG₄/IgG₁) with a change in conjunctival sensitivity within the variation demonstrated in the control group. In addition, 3 other patients with decreased conjunctival sensitivity showed a subclass ratio well below 0.5. The patients in the control group showed a very low antibody ratio and conjunctival

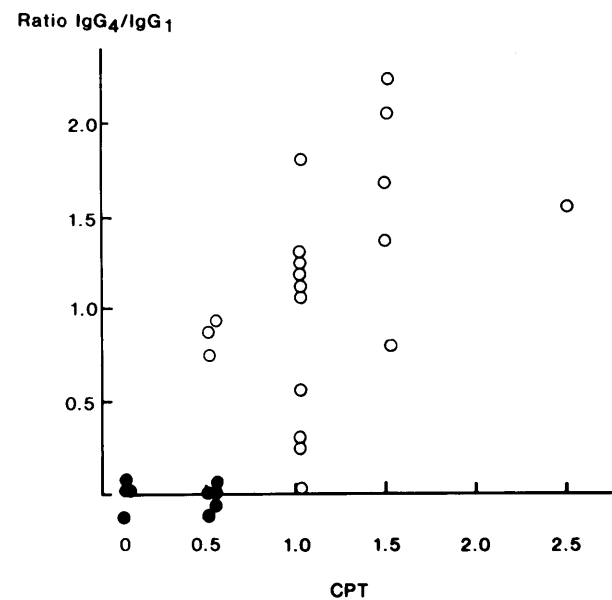


Fig. 5. Changes in the subclass ratio IgG₄/IgG₁ to a house dust mite allergen, *Der f II*, versus changes in the conjunctival sensitivity tested with *D. farinae* allergens after a treatment period of 12 months. Treated patients (○); control (●).

sensitivity within half a 10 log step. The correlation between the IgG subclass ratio (IgG₄/IgG₁) and clinical improvement as measured by decreased conjunctival sensitivity was significant at the 0.05 level.

Discussion

A purified, standardized and freeze-dried *D. farinae* allergen preparation was used for immunotherapy in 16 adults with documented allergy to house dust mite (16). There was a significant improvement in clinical parameters of the treated patients, who tolerated high concentrations of *D. farinae* allergens in skin prick test and conjunctival provocation test and improved subjectively in comparison with open controls after treatment for 1 year. The antibody response showed an initial increase for IgE followed by a rather fast decrease. Thus, the specific IgE antibody level after treatment was well below the pretreatment value. IgG-RAST for *D. farinae* allergen indicated a slow initial increase to a plateau value followed by a slow decrease, but after 1 year the specific IgG antibody level was still significantly above the pretreatment level. These patterns are in accordance with most other studied allergens (20). Generally, specific IgG increase during IT, but in one study by Karlsson et al. (15) the level of *Cladosporium herbarum*-specific IgG antibodies decreased significantly after 3 months of therapy, and after 10 months the specific IgG antibody level was approximately the same as the pretreatment level.

The results presented for house dust mite-specific IgG subclass antibodies confirm that the humoral response to mite allergens during immunotherapy is restricted to the production of IgG₁ and IgG₄ antibodies. It is well known that the main response to stimulation with protein antigen is production of IgG₁ subclass antibodies, while repeated exposure favors antibodies of the IgG₃ and IgG₄ subclasses (13), and during repeated administration of allergens the IgG₄ antibody response dominates (10). The increase in *D. farinae* mite-specific IgG₄ antibody was pronounced and still increasing after 12 months of active treatment. This effect was observed for both *D. farinae* allergens and the isolated allergen *Der f II*. Similar findings have been reported by Nakagawa et al. (22, 27) in patients undergoing immunotherapy with house dust mite *D. farinae*. In several immunotherapy studies with *Hymenoptera* insect venoms and grass pollens a drastic rise in specific IgG₄ antibodies has been noted (1, 2, 11, 32). It has been suggested that allergen-specific IgG₄ antibodies induced during immunotherapy may play a blocking or protective role in IgE-dependent immediate allergic reactions (1, 23, 32). Further, significant correlation was observed between IgG₄ and total IgG antibody levels but not between IgG₄ and IgE antibody levels during immunotherapy with *D. farinae* allergens (24).

IgG₄ has also been suggested as a marker of allergy, but allergen-specific IgG₄ antibodies may also be found in non-allergic individuals, which does not fit that hypothesis (33). Others have suggested that IgG₄ is simply a marker of antigen exposure. Djurup et al. (7–9) have proposed that a too early and intense increase of specific IgG₄ or a late high IgG₄ antibody level may lead to a poor clinical outcome of immunotherapy in patients with inhalant allergy. Similar findings have also been reported by Etievant et al. (12). A rapid and pronounced increase of IgG₄ antibodies was noted during the initial phase of treatment and this would suggest treatment failure according to data by Djurup et al. (8). However, our data indicate that the levels of mite-specific IgG₄ subclass antibodies were associated with a positive clinical effect. The reason for this discrepancy might be the fact that mites and pollen differ with respect to antibody induction, i.e. house dust mites act as a perennial allergen while pollens are seasonal, or that adults and children may respond differently to immunotherapy.

Immunotherapy studies with venoms or grass pollen extracts have suggested that a vigorous IgG₁ response might result in feed-back inhibition of IgE synthesis and thus represents a protective effect (7). In this study IgG₁ peaks in most patients after 6 months of treatment, followed by a marked decrease. But at termination of the immunotherapy after 12

months the allergen-specific IgG₁ antibody levels were still significantly higher compared with pre-treatment level. The magnitude of the mite-specific IgG₁ response during the dose-increase of IT correlated to changes in IgE parameters (16). Thus, the covariation between mite-specific IgG₁ and IgG₄ antibodies might indicate their role as indicators of clinical improvement.

Based on the fact that the level of IgG₄ antibodies in patients with atopic dermatitis was higher for perennial than for seasonal allergens, it might be envisaged that chronic exposure enhances the development of IgG₄ response (2). Repeated administration of high doses of allergen during immunotherapy might result in a drastic increase in IgG₄ levels. This is also the case in this study since a marked increase in allergen-specific IgG₄ levels could be demonstrated. Specific IgG₄ antibodies against food antigens have been repeatedly demonstrated (31). By contrast, the development of IgG₄ responses to inhalant allergens in patients naturally exposed may be delayed up to 2 years after demonstration of IgG response, most likely reflecting the lower exposure to mite and grass pollen allergens.

The ratio IgG₄/IgG₁ showed a distinct increase in the active group both for the isolated *Der f II* allergen and *D. farinae* allergen mixture, while in controls it remained constant at the pretreatment level. A mean rise of 3.4-fold in the ratio IgG₄/IgG₁ (*D. farinae* allergen mixture) and 2.6-fold for *Der f II* was obtained after 12 months treatment. However, already after 3 months there was a significant difference between the active group and control. According to Djurup & Østerballe (9) an IgG₄/IgG₁ ratio > 1.0 was associated with a poor therapeutic outcome for grass pollen-treated patients. This is in contrast to our house dust mite data where we had a positive clinical outcome in patients given immunotherapy with *D. farinae* mite extract, as the median ratio IgG₄/IgG₁ was well above 1.0. In another immunotherapy study using *Cladosporium herbarum* the IgG subclass response showed no relation of IgG₄/IgG₁ ratio to changes in IgE-mediated parameters (17). The authors advocated that the subclass ratio was not a good prognostic indicator of clinical efficacy in mould immunotherapy. The ratio IgG₄/IgG₁ for *Der f II* versus CPT demonstrated a high correlation of clinical improvement, whereas in controls the threshold concentration and IgG₄/IgG₁ ratio did not change during the treatment period, supporting the use of *in vitro* parameters as indicator for clinical efficacy.

The present study has demonstrated that a sensitive ELISA method is suitable for measuring mite-specific IgG subclasses in patients undergoing immunotherapy. However, the change in these *in vitro* parameters was only 3–6 fold, whereas the change

in skin sensitivity was 10–1000 fold and the change in conjunctival sensitivity 3–300 fold. This does not disallow that the allergen-specific IgG₄/IgG₁ ratio, using an isolated allergen component, can be used for monitoring the clinical outcome of immunotherapy.

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